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This project proposed the development of a recombinant polyclonal antibody library specific for the human breast carcinoma cell line BT-20. The proposed library would target multiple epitopes on the human cell line and also provide the genes necessary for the unlimited perpetuation of the defined antibody mixture. Ultimately, such antibody libraries could be used for treatment and diagnosis of breast cancer in humans. In the present study, an Fab phage display library specific for the BT-20 cells has been generated. To obtain a polyclonal antibody library of whole IgG antibodies that would mediate effector functions, a vector system for mammalian expression has been constructed. The new system uses restriction enzyme-based transfer of variable region gene pairs from a phage display to a mammalian expression vector. Improved methods for handling antigen-selected Fab phage display library were also developed. Construction of a new anti-BT-20 library, using the improved vectors and methods, has begun.

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INTRODUCTION:

As an immunotherapeutic approach to breast cancer, we set out to develop recombinant polyclonal antibody libraries (PCALs) that would target malignant cells. PCALs are standardized mixtures of polyclonal antibodies that can be perpetuated and modified as desired. Hence, they combine the advantages of targeting multiple antigenic determinants (low likelihood of antigen 'escape variants') with the advantages of using monoclonal antibodies (unlimited supply of standardized reagents, and the availability of the genetic material for desired manipulations).

Our laboratory has developed a system for generation of PCALs (1, 2). This system involves the cloning of VL-VH region gene pairs into a bidirectional phage display vector for Fab expression, such that the VL and VH region genes of each pair are in opposite (head to head) transcriptional orientations ($\leftarrow\rightarrow$). Because the phage display vector is circular, the vector library can be opened with restriction enzymes between VL and VH to insert a bidirectional leader-promoter-promoter-leader (*lPPl*) cassette that allows expression of Fab on the phage surface. Furthermore, a subpopulation of Fab-phage can be selected for binding to a desired antigen or polyantigen (a multi-antigen particle such as a mammalian or microbial cell). The VL-VH region gene pairs of the selected phage subpopulation are then transferred, in mass, from the phage display vector to a bidirectional mammalian expression vector to produce whole antibodies that can mediate effector functions.

BODY:

Using the system we developed for PCAL production, we generated an Fab phage display library to the human breast carcinoma cell line BT-20, from the expressed variable region genes of an immunized mouse. We reported our results with the Fab phage display library to the human breast carcinoma cell line BT-20 in appended publication #1. The antigen-selected library was shown to bind specifically to BT-20 cells. However, an attempt to transfer the selected heavy and light chain variable region gene pairs -- proved unsuccessful, although the vector system had worked well with the simpler model system (2). We therefore turned to development of improved vectors and methods.

The original protocol included a mammalian vector for expression of mouse IgG2b antibodies. This vector, denoted pMDV- γ 2b (Murine Dual Vector- γ 2b) contains the mouse γ 2b heavy chain gene and the mouse κ light chain gene in opposite transcriptional orientations. A diagram of this vector is shown in Fig. 1. VL-VH region genes linked head to head (\longleftrightarrow) in opposite transcriptional orientations (and containing a mammalian cassette with promoter and leader sequences [*lPPI* cassette]) could be lifted by PCR from the phagemid vector and cloned between the EcoRI and HindIII sites.

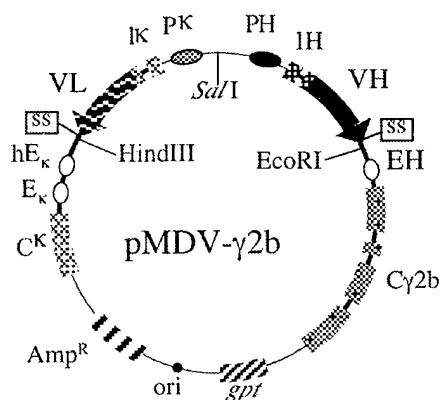


Fig. 1 Bidirectional mammalian vector for expression of antibodies with mouse IgG2b C regions. Exons are boxed. Transcription regulatory elements are oval. Unless otherwise indicated, all genes and regulatory sequences are of murine origin. EH, EK, and EK hum are the H chain, the κ chain, and the human κ chain enhancers respectively; PH and PK are the H chain and κ chain promoters; lH and lK are the H chain and κ chain leader sequences; VH and VK represent the rearranged VH region and VK region genes. ss, splice site; *amp^r*, the ampicillin resistance gene; *ori*, origin of DNA replication of pBR322; *gpt*, the *E. coli* gene that encodes the xanthine-guanine phosphoribosyltransferase enzyme (a selectable marker in transfections). The vectors are not drawn to scale.

Because of the inefficiency of the PCR-based transfer of variable regions genes from anti-carcinoma Fab phage display libraries using the original system, and also to prevent possible bias in PCR amplification of certain clones in a PCAL, we constructed a cassette-providing vector (p/P-EH-P/) and a constant region providing vector (pMDV-IgG2b). Fig. 2 shows the construction schemes of these two vectors.

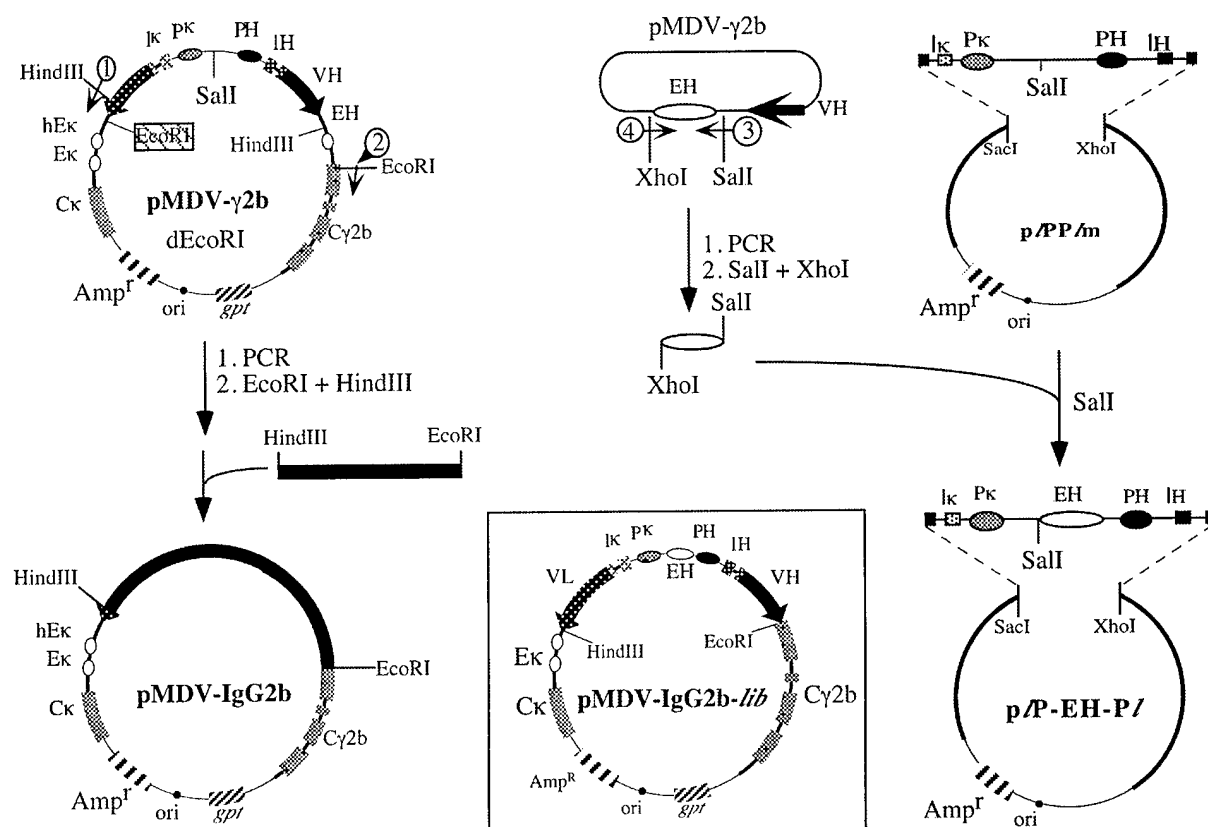


Fig. 2. Schemes of generation of the modified mammalian cassette promoter providing plasmid p/P-EH-P/ and backbone providing pMDV-IgG2b vector. Primers are designated by arrows. The final expression plasmid is also shown (boxed).

The pMDV-IgG2b backbone was generated by PCR using the GeneAmp XL-PCR Kit (Perkin-Elmer), which is designed to amplify >20 kb DNA fragments with high fidelity (3, 4). The template for the long PCR was a modified pMDV-γ2b (Fig. 1) in which the EcoRI site downstream of VL was destroyed by EcoRI digestion and Klenow fill-in (pMDV-γ2b-dEcoRI). A HindIII or EcoRI site was incorporated into primers 1 and 2, respectively, at positions which coincide with the phage vector. Primer 1 contains the sequence corresponding to codons 110 to 108 of the V_k and 11 nucleotides of the J_k-C_k

intron. The HindIII site was created in primer 1 by changing the third nucleotide of codon 114 without changing the encoding amino acid. Primer 2 contains the sequence corresponding to the first 11 codons of the Cy2b. The 2nd to 4th amino acid residues were changed from Lys to Asn, Thr to Ser, and Thr to Lys, respectively. The 2nd and 3rd amino acid residues changed were a result of introducing an EcoRI site. The changing of the 4th amino acid residue was to prevent the creation of a potential N-linked glycosylation site.

It should be noted that the EcoRI and HindIII sites switched in pMDV-IgG2b (from the previous pMDV- γ 2b vector) but also the 1.5 kb DNA fragment containing the EH was deleted. To provide the enhancer activity required for PH function and to ultimately reduce the size of the final expression plasmid, a DNA fragment containing the EH (~ 300bp) was inserted upstream of PH to obtain vector p/P-EH-Pl. The EH was first amplified from vector pMDV using primers 3 and 4 which contained SalI and XhoI sites, respectively. The resulting PCR products were digested with SalI and XhoI, ligated with SalI linearized p/PP/m vector (see Fig. 2), and transformed into *E. coli*. Because SalI and XhoI are isoschizomers, the XhoI site was destroyed after ligation. Plasmids containing the EH fragment were identified by SacI and XhoI double digestion. The orientation of EH could also be verified by SalI/SacI or SalI/XhoI double digestion.

Thus, the pMDV-IgG2b and pLPEHPl vectors allow the direct cloning of the selected VL-VH pairs between phage display and mammalian expression systems. The functionality of the modified bidirectional vectors were tested by expression of monoclonal antibody with 36-65 V- regions, which are specific for *p*-azophenylarsonate (Ars). The affinity of the resulting antibody for Ars was similar to the original 36-65 antibody. The results suggested that the amino acid substitutions in the pMDV-IgG2b had little or no effect on antigen recognition.

The improvement in generation of polyclonal IgG2b using this modified system was also examined. The mouse IgG expression library was generated by mass transfer of the linked VL-VH pairs from a mouse Fab phage library. The final expression plasmids were transfected into Sp2/0 null hybridoma cells by spheroplast fusion. The

transfected cells were plated into 96-well plates with 10^5 cells/well. Multiple clones were observed in the individual wells. The percentage of Ig producers was determined to be 75 to 85% by sandwich ELISA using goat anti-mouse IgG2b as the capture Ab and detection by alkaline phosphatase conjugated anti-mouse IgG. The production of intact Ig was confirmed by Western blotting.

The use of the plpEHP1 and MDV-IgG2b vectors for PCAL generation was reported in appended publication #2.

The improved vector system was used to proceed with the construction of the anti-breast cancer PCAL, starting with the anti-BT-20 Fab phage display library. In the course of experiments, we found that repeated propagation of the selected anti-BT-20 phage library resulted in loss of binding activity, and that we no longer had an active library. This is presumably due to overgrowth by library members that do not express full Fab molecules and hence have a growth advantage.

To deal with this problem, we improved our system for handling selected phage libraries by freezing phage-harboring bacterial stocks in aliquots in 25% glycerol at -80°C immediately after selection. To make phage, one aliquot at a time is defrosted followed by plating of the bacteria on glucose-containing agar plates and scraped bacteria are then used for phage production.

We have begun the production of a new anti-breast cancer phage library from a BT-20-immunized mouse, and this library will be processed to produce an anti-breast cancer PCAL using the improved vectors and methods.

KEY RESEARCH ACCOMPLISHMENTS

- Mice were immunized with human breast carcinoma cell line BT-20
- An original Fab phage library was generated from the V region genes obtained from an immune mouse
- An Fab phage display library was generated
- The Fab phage display library was positively selected on the BT-20 human breast carcinoma cell line, and shown to be specific and polyclonal

- An improved mammalian expression vector system was generated that allows efficient mass transfer of variable region gene pairs between phage display and mammalian expression vectors
- The functionality of the improved system was demonstrated
- Improved methods for handling selected phage libraries were developed
- Construction of a new anti-BT-20 library, using the improved vectors and methods, has begun

REPORTABLE OUTCOMES

Publications:

1. Santora, K. E., Sarantopoulos, S., Den, W., Petersen-Mahrt, S., Sompuram, S. R., and Sharon, J. (2000) Generation of a polyclonal fab phage display library to the human breast carcinoma cell line BT-20. *Comb Chem High Throughput Screen* **3**, 51-57.
2. Sharon, J., Sarantopoulos, S., Den, W., Kao, C.-Y., Baecher-Allan, C. M., Santora, K. E., Sompuram, S., Petersen-Mahrt, S., and Williams, B. R. (2000) Recombinant polyclonal antibody libraries. *Comb. Chem. High Throughput Screen.* **3**, 185-196.
3. Sharon, J., Santora, K., Sarantopoulos, S., Den, W., Petersen-Mahrt, S., and Sompuram, S. (2000) Generation of a polyclonal Fab phage display library to the human breast carcinoma cell line BT-20. Era of Hope, Department of Defense Breast Cancer Research Meeting, June 8-11, Atlanta, Georgia, Proceedings p 645.

CONCLUSIONS

In the present study, V region genes for construction of a polyclonal Fab phage display library were obtained from the spleen, bone marrow, and intestine of a Balb/c

mouse that had been immunized with BT-20 cells. The light and heavy chain V region genes were linked by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into a bidirectional phagemid vector to express an Fab-displaying phage library. This library was then selected for binding to BT-20 cells. Direct and inhibition ELISA showed that the selected Fab phage display library bound specifically to BT-20 cells, and that this binding could be inhibited by BT-20 as well as by other human breast cancer cell lines, but not by sheep red blood cells. These results demonstrate that the first phase of generating a polyclonal antibody library can be done successfully.

An improved mammalian expression vector system has been generated to attain efficient mass transfer of variable region gene pairs from phage display to mammalian expression vectors for expression of polyclonal IgG antibody libraries. In addition, improved methods for handling antigen-selected Fab phage display libraries were developed. Construction of a new anti-BT-20 library, using the improved vectors and methods, has begun.

Recombinant polyclonal antibody libraries could be used for treatment and diagnosis of breast cancer in humans.

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APPENDIX

1. Santora, K. E., Sarantopoulos, S., Den, W., Petersen-Mahrt, S., Sompuram, S. R., and Sharon, J. (2000) Generation of a polyclonal fab phage display library to the human breast carcinoma cell line BT-20. *Comb Chem High Throughput Screen* **3**, 51-57.
2. Sharon, J., Sarantopoulos, S., Den, W., Kao, C.-Y., Baecher-Allan, C. M., Santora, K. E., Sompuram, S., Petersen-Mahrt, S., and Williams, B. R. (2000) Recombinant polyclonal antibody libraries. *Comb. Chem. High Throughput Screen.* **3**, 185-196.
3. Sharon, J., Santora, K., Sarantopoulos, S., Den, W., Petersen-Mahrt, S., and Sompuram, S. (2000) Generation of a polyclonal Fab phage display library to the human breast carcinoma cell line BT-20. Era of Hope, Department of Defense Breast Cancer Research Meeting, June 8-11, Atlanta, Georgia, Proceedings p 645.

Generation of a Polyclonal Fab Phage Display Library to the Human Breast Carcinoma Cell Line BT-20

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Abstract: We have previously described a vector system for generating recombinant polyclonal antibody libraries. This system uses bidirectional phagemid and mammalian expression vectors to facilitate mass transfer of selected variable light and variable heavy (VL-VH) region gene pairs from the phagemid to the mammalian vector, to express polyclonal libraries of whole IgG antibodies. We report here the first stage of generating a polyclonal antibody library to the human breast carcinoma cell line BT-20, using this vector system. VL and VH region gene pairs were obtained from a mouse immunized with BT-20 cells, and cloned, in opposite transcriptional orientations, in the bidirectional phagemid vector, to produce an Fab phage display library. This library was selected by panning on BT-20 cells and shown to bind specifically to BT-20 cells. Such libraries, after suitable negative selection to eliminate major reactivities against normal tissue, could be transferred in mass to our bidirectional mammalian expression vector for production of libraries of chimeric antibodies with mouse V regions and human constant (C) regions. These polyclonal antibody libraries will mediate effector functions and are expected to be useful for breast cancer therapy, as well as diagnosis.

Introduction

As an immunotherapeutic approach, we set out to develop a recombinant polyclonal antibody library that would target human breast cancer cells. Polyclonal antibody libraries (PCALs, pronounced "P"-Cals") are standardized mixtures of whole antibodies that can be perpetuated and modified as desired because the antibody genes are available. Therefore, they combine the advantages of conventional polyclonal antibodies (targeting of many antigenic determinants, low likelihood of antigen 'escape variants', and efficient mediation of effector functions) with the advantages of monoclonal antibodies (unlimited supply of standardized reagents, and the availability of the genetic material for desired manipulations).

We have previously described a system for generation of recombinant polyclonal antibody libraries [1,2]. This system involves the cloning of VL-VH region gene pairs into a bidirectional phage display vector for Fab expression, such that the VL and VH region genes of each pair are in opposite, head to head, transcriptional orientations. Because the phage display vector is circular, the vector library can be opened with

restriction enzymes between VL and VH to insert a bidirectional leader-promoter-promoter-leader (I/P/I) cassette that allows expression of Fab on the phage surface. Furthermore, a subpopulation of Fab-phage can be selected for binding to a desired antigen or polyantigen (a multi-antigen particle such as a mammalian or microbial cell). The VL-VH region gene pairs of the selected phage subpopulation are then transferred, in mass, from the phage display vector to a bidirectional mammalian expression vector to produce whole antibodies (PCALs) that can mediate effector functions. Regarding future FDA approval, PCALs should fall under the same category as such clinically used polyclonal antibody products as human gamma globulin preparations, anti-thymocyte globulin (ALG) and anti-thymocyte sera (ATS) [3,4]. Like these products, every PCAL batch will have to be tested for safety, but PCALs are expected to show much less batch-to-batch variability.

In the present study, we have used a bidirectional I/P/I cassette containing the Zeocin™ selectable marker [5,6] to achieve efficient expression of Fab-displaying phage for generating a specific polyclonal Fab phage display library to the human breast carcinoma cell line BT-20.

Other studies have shown that the phage display system is a powerful tool for picking out anti-cancer antibodies [7-12]. Cai *et al* [10,11] were able to isolate

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specific anti-melanoma monoclonal antibodies from melanoma patients immunized with genetically modified autologous tumor cells, and Figini *et al* [8] isolated human Fab fragments from phagemids against ovarian carcinoma.

A polyclonal antibody library against a malignant subpopulation of human cells would be intended to target those unique antigens that might be expressed only on cells of the subpopulation but not on other cells in the individual or species, as well as antigens that are expressed preferentially on cells of the subpopulation. It has been shown that there are variations in expression of antigens between malignant cells and normal breast epithelial cells [13]. Some tumor-associated antigens were shown to (a) be expressed at a reduced level in malignancy (integrins, suppressor genes *p53* and *Rb*); (b) have an increased level of expression (protooncogenes *c-erbB2*, *EGF-R*, *c-Hras*, *c-myc* and polymorphic epithelial mucin [PEM]); or (c) be distinct from the normal antigen due to genotypic (*p53*) or phenotypic (PEM) changes. For example, there is aberrant glycosylation of PEM in breast cancer cells [14,15]. We intend to take advantage of these differences in antigenic profile between normal and malignant breast cells in the development of polyclonal antibody libraries.

Materials and Methods

Mammalian Cells

The human breast carcinoma cell line BT-20 was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and was maintained in culture in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), at 37°C, in a humidified atmosphere of 5% CO₂ / 95% air. Sheep red blood cells (SRBCs) were obtained from PML Microbiologicals (Tualatin, OR), and kept at 4°C until use (not more than 30 days).

Immunization of Mice

BALB/c female mice (8-12 weeks old, from Charles River Laboratories, Cambridge, MA) were immunized intraperitoneally (i.p.) with 5 x 10⁶ live BT-20 cells that had been washed with phosphate buffered saline (PBS, Gibco BRL) and scraped into 200 µl of PBS. The mice were boosted i.p., subcutaneously (at the base of the tail), and intravenously, on day 30 post primary immunization, with a total of 1 x 10⁶ BT-20 cells in 100 µl saline. The mouse showing the highest antibody titer was boosted again, i.p. and subcutaneously, on day 44

post primary immunization with 1 x 10⁶ BT-20 cells in 100 µl saline and sacrificed 5 days later.

Construction of a Fab Phage Display Library

RNA was prepared from the spleen, intestine, and bone marrow of the BT-20-immunized BALB/c mouse using the Ultraspec™-II kit (Biotecx Laboratories Inc., Houston, TX) according to the manufacturer's instructions.

Preparation of head to head linked VL-VH region gene pairs, by reverse transcription - polymerase chain reaction (RT-PCR) and overlap extension PCR, was performed by the previously described scheme [2], using the primers indicated in Fig. (1). SuperScript™ reverse transcriptase (Gibco BRL) was used for first strand cDNA synthesis and Taq DNA polymerase (Gibco BRL) was used for all PCRs. Four RT reactions were done for each tissue, one with each of the following primers: CL-κ-RT, CH1-μ-RT, CH1-γU-RT, and CH1-α-RT.

First strand cDNA (RT) primers (as)

CH1-μ-RT ¹⁶⁶ GAT GAC TTC AGT GTT GTT CTG GTA GTT CC
CH1-γU-RT ¹⁹⁶ GCT GGA GGG TAC AGT CAC TGA GGT GCT
CH1-α-RT ¹⁵⁷ CCA GGT CAC ATT CAT CGT GCC GAA AGG GAA G
CL-κ-RT ¹⁴⁹ TT CCA CTT GAC ATT GAT GTC TTT GGG GTA G

First PCR forward primers (sn)

VH1-link ^{SacI} 30N linker ¹ GAG CTC 24N ^{XhoI} GAG GTG CAG CTT CTC GAG TCA GGA CCT GGC CT
VH2-link ^{SacI} 30N linker ¹ GAG CTC 24N ^{XhoI} GAG GTG CAG CTT CTC GAG TCT GGG GCT GAG CT
VH3-link ^{SacI} 30N linker ¹ GAG CTC 24N ^{XhoI} GAG GTG CAG CTT CTC GAG TCT GGG GGAGGCT
VL1-link ^{SacI} 30N linker ³ 24N GAG CTC ^{XhoI} GTG ATG ACA CAG ACT CCA TCC TCC CTG CCT G
VL2-link ^{SacI} 30N linker ³ 24N GAG CTC ^{XhoI} GTT CTC ACC CAA TCT CCA GCA TCA ATG TCT G

First PCR reverse primers (as)

CH1-μ ¹²⁵ GAC GAG GGG GAA GAC ATT TGG GAA GGA CTG
CH1-γ ¹⁵¹ GGC TCA GGG AAG TAI CCI TTG ACC AGG CA
CH1-α ¹²⁵ TGT CAG TGG GTA GAT GGT GGG ATT TCT CGC
CL-κ ¹²¹ GGA TGG TGG GAA GAT GGA TAC AGT TGG TGC

30 N Linkers

VH ^{5'} GAG CTC TTC TCT AAC GAT TAC CTA TCT AGC ^{3'}
VL ^{3'} CTC GAG AAG AGA TTG CTA ATG GAT AGA TGC ^{5'}

Second (nested) PCR primers (as)

JL-κ1 ¹¹⁰ CG TTT GAT TTC AAG CTT GGT GCC TCC ACC ⁹⁹
JL-κ2 ¹⁰⁸ TTT TAT TTC AAG CTT GGT CCC CCG TCC GAA CGT ⁹⁷
JL-κ5 ¹⁰⁸ ATC AGC CCG TTT TAT TTC AAG CTT TGT CCC CGA GCC GAA CGT
JH-1 ^{EcoRI} ¹¹³ CTT AGA ATT CGC TGA GGA GAC GGT GAC CGT GGT CCC TGC GC
JH-2 ^{EcoRI} ¹¹³ TC GGA ATT CGC TGA GGA GAC TGT GAG AGT GGT GCC
JH-3 ^{EcoRI} ¹¹³ CTT AGA ATT CGC TGC AGA GAC AGT GAC CAG AGT CCC TTG GC
JH-4 ^{EcoRI} ¹¹³ CTT AGA ATT CGC TGA GGA GAC GGT GAC TGA GGT TCC TTG AC

Fig. (1). Oligonucleotide primers used for library construction. The antisense and sense strands are denoted by "as" and "sn", respectively. Restriction sites are underlined. Corresponding amino acid numbers in the Kabat system [25] are shown above the sequences. Linker regions (the 30 nucleotide tails plus the first nucleotide of the VL or VH region) are indicated. N, nucleotide(s).

The cDNAs from each tissue were then amplified in 11 separate low-stringency PCRs (37°C annealing temperature). The κ chain cDNA was amplified in two reactions with the CL- κ reverse primer and either VL-1-link or VL-2-link forward primers [see Fig. (1)]. Each of the three H chain cDNAs (μ , γ , and α) was amplified in three reactions, all with the CH1- μ , CH1- γ , or CH1- α reverse primers for the μ , γ , and α cDNAs respectively, and either the VH1-link, VH2-link, or VH3-link forward primers [Fig. (1)]. The VL-link and VH-link primers have complementary 5' tails that allow subsequent hybridization and overlap extension to link L and H chains in pairs.

The two L chain PCR products and the nine H chain PCR products, obtained from each tissue, were separately gel-purified from a 1.2% agarose gel via the GeneClean II kit (Bio 101, La Jolla, CA). All L chain PCR products were then combined, resulting in a single L chain sample per mouse. The nine H chain products obtained from each tissue were combined, resulting in one H chain sample per tissue (a total of four samples). Each of the four H chain samples were combined with the L chain sample in an overlap extension reaction with no additional primers for 12 cycles at 94°C 10 sec, 59°C 30 sec, 72°C 30 sec.

The four overlap reactions were then combined and used as template for a "Nested PCR" in 16 separate reactions, each reaction containing a different combination of one of four JL- κ primers and one of four JH primers, as described [2], see Fig. (1) for primer sequences]. Each JL- κ primer contains a *Hind* III site and each JH primer contains an *Eco*R I site to allow cloning of the PCR products. The resulting products (approximately 740 bp) were gel-purified from 0.8% agarose gels, combined, digested with *Eco*R I/*Hind* III, gel-purified again, and ligated with the *Eco*R I/*Hind* III-digested and gel-purified 4.1 kilobasepairs (kb) backbone from phagemid vector #541 phh3mu-y1 [2]. All DNA fragments used in subsequent ligations for library construction were gel-purified.

The VL-VH region gene pairs were ligated into the phh3 vector [2] to generate the phh3-VL-VH library of vectors [see Fig. (2)]. Bacterial transformation and amplification of the phage library was done according to Barbas and Lerner [16]. Briefly, the library was used to transform, by electroporation, competent XL1-Blue bacteria [XL-1 Blue electrocompetent cells (Stratagene, LaJolla, CA) transformation efficiency 5×10^9 colony forming units (cfu) per mg DNA], and plated at high density on plates containing carbenicillin (50 μ g/ml) and tetracycline (10 μ g/ml). In addition, aliquots of the transformed cell culture were plated at low density to determine the library size (the number of original members) [17]. The colonies were scraped off the high density plates and double stranded phagemid DNA was prepared by the QIAprep Spin miniprep kit (Qiagen Inc., Valencia, CA) and digested with *Sac*I and *Xho*I to generate a 4.8 kb backbone. This backbone was ligated with a 0.9 kb fragment containing head to head promoter and signal sequences as well as the Zeocin™ selectable marker (Invitrogen, San Diego, CA, [5,6]), from plasmid #560 p/PZeoPI [see Fig. (2)]. Vector #560 had been created (Santora and Sharon, manuscript in preparation) by PCR-lift of the Zeocin™ resistance gene (*Zeo*), including the small synthetic prokaryotic promoter EM-7, from the pZeoSV2(+) Invitrogen vector and ligation into the previously described #506 p/PPI vector [2]. The resulting phh3-VL-IPZeoPI-VH library [see Fig. (2)] was used to transform XL1-Blue bacteria and the library was plated at high density on plates containing 5 μ g/ml Zeocin™.

For preparation of phage, the high-density bacterial plates were scraped into carbenicillin- and tetracycline-containing super broth (SB) and infected with excess helper phage VCSM13 [Stratagene, 10^{12} plaque forming units (pfu)] for 2.5 hr at which time the culture was grown 5 more hr with the addition of kanamycin (70 μ g/ml). Phage were precipitated from the cell supernatant with polyethylene glycol (1:5 dilution of 27% PEG/3.3 M NaCl), spun out at 12,000 rpm in a

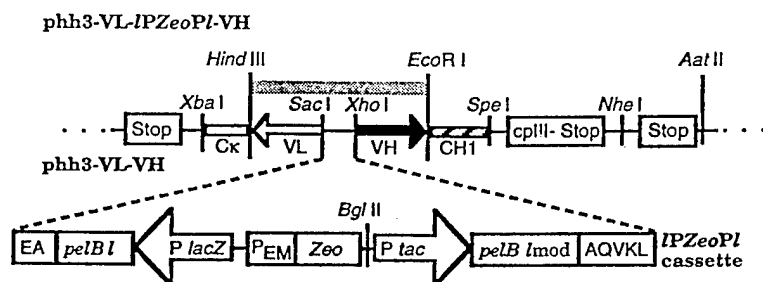


Fig. (2). Diagram of VL-VH region gene library in phage display vector (partial map and not to scale). The vector is circular. The positions of restriction enzyme sites are indicated by vertical lines. P, promoter; I, leader; Stop, translation termination. VL and VH region N-terminal amino acids provided by the promoter-leader cassette are shown in one letter code. The *Zeo* gene encodes resistance to Zeocin™. The arrows representing the VL and VH region genes and the promoters indicate the transcriptional orientation.

Sorvall SS-34 rotor for 20 min, and resuspended in PBS. This was the 'original' Fab phage library.

Selection of the Fab Phage Display Library on BT-20 Cells

The Fab phage display library was positively selected for reactivity with BT-20 cells by panning: one ml of phage (containing 10^{11} cfu) were pre-absorbed on individual wells of a 24-well plate, first on a well coated with 20 μ g/ml bovine serum albumin (BSA), and then on a well coated with 20 μ g/ml collagen. (This treatment was done in an attempt to deplete phage that would bind to blocking agents in the subsequent panning on BT-20 cells.) The absorbed library was then rocked for 3 hr at room temperature on BT-20 cells that had been plated in a 25-cm² tissue culture flask, grown to confluence, and fixed with 4% paraformaldehyde as described [16,17]. The flask was washed 10X with buffer (1% Ovalbumin/0.05% Tween 20/0.5 M NaCl in PBS), and bound phage were eluted from the fixed cells with 0.1 M glycine/HCl pH 2.2 containing 1 mg/ml BSA as described [16]. After 5 min, 6 ml of 2 M Trizma base was added for neutralization, followed by 10 volumes of SOC medium [18]. The cell supernatant containing the eluted phage was used to infect XL1-Blue electrocompetent bacterial cells in log phase, and aliquots removed from the transformed cell cultures for plating to determine the number of eluted phage. The cultures were plated onto carbencillin-containing LB plates, the colonies were scraped, and used to prepare double-stranded phagemid DNA and phage, as described above. The panning procedure was repeated only once in order to maintain a sufficient polyclonal population.

Membrane Preparations

Membranes were prepared from BT-20 cells as described [19]. The membrane preparations were stored in Eppendorf tubes in aliquots containing 1 mM of each of the following protease inhibitors: leupeptin, aprotinin, pepstatin, and PMSF (from Boehringer Mannheim Corp., Indianapolis, IN), in phosphate buffered saline (PBS, 2.3 mM NaH₂PO₄/9.7 mM Na₂HPO₄/150 mM NaCl, pH 7.2) at -80°C.

SRBC ghost membrane preparations were obtained by hypotonic shock of SRBCs as described [20]. Ghosts were stored in Eppendorf tubes in PBS at -20°C in aliquots containing the same protease inhibitors used for BT-20 membrane preparations.

Protein concentrations of membrane preparations were determined by the Bradford assay [21] using the Bradford Reagent (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

ELISA

Antibody titers on sera obtained from immunized mice were determined by ELISA using 96-well microtiter plates (Corning #25805-96, Corning, NY) coated with BT-20 cells fixed in 4% paraformaldehyde for 15 minutes as described [16,17]. The ELISAs were developed with alkaline phosphatase (AP)-conjugated antibodies specific for mouse Ig (Sigma Chemical Co.), and after washing, 100 μ l per well of AP substrate was added; AP substrate was made by adding one tablet of *p*-Nitrophenyl Phosphate, Disodique (Sigma) to 5 ml DEA solution (diethanolamine, Fisher Scientific, Fair Lawn, NJ). The reaction was stopped with 0.1 M EDTA and absorbance read at 405 nm.

For phage ELISA, plates were coated with 20 μ g/ml of membranes in PBS overnight at 4°C. Plates were then washed 2X with PBS and blocked with buffer (1% Ovalbumin/0.05% Tween 20/0.5 M NaCl in PBS) for 1 hr at room temperature. Phage were added (at 1:3 serial dilutions starting at 10^{11} cfu) and incubated with light rotation for 3 hr at room temperature. The plates were washed 10X in buffer, 5 minutes per wash, with high rotation. Primary antibody was added, biotinylated sheep anti-M13 (5'-3' Inc, Boulder, CO) 1:1000 in 1% BSA/PBS, and incubated 1 hr at 37°C. The plates were then washed 6X in PBS/0.05% Tween 20, 5 min per wash, with high rotation, and the secondary antibody was added, AP-conjugated streptavidin (Pierce, Rockford, IL), at 1:2500 in 1% BSA/PBS for 30 min at 37°C. Plates were washed three times with PBS/0.05% Tween 20, and developed with AP substrate as described above.

Inhibition ELISA with Fab phage display libraries was performed as described for direct ELISA with the following exception: before addition of 10^{11} cfu of phage to each well, the phage were incubated with 0 μ g/ml, 0.1 μ g/ml, 1.0 μ g/ml or 4.0 μ g/ml of membrane preparation for 1 hr.

Results and Discussion

The human breast adenocarcinoma cell line BT-20 was chosen for generation of an anti-human breast cancer polyclonal antibody library (PCAL) because of the ability of BT-20 cells to form tumors in immunodeficient nude mice [22]. This affords a system for *in vivo* efficacy testing of the library. V region genes for construction of a polyclonal Fab phage display library to BT-20 cells were obtained from a BALB/c mouse that had been immunized with BT-20 cells. The serum antibody titer was assessed, by ELISA on fixed BT-20 cells, at various days post primary immunization. As shown in Fig. (3), the serum titer rose to almost 17,000 on the day of sacrifice (day 49 post primary immunization, 5 days after the second boost).

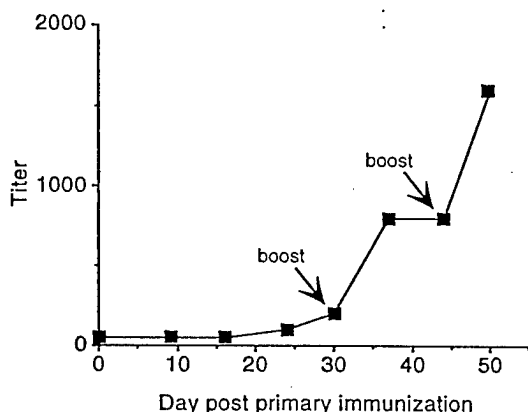


Fig. (3). Serum antibody response of the BT-20-immunized mouse used for library construction.

Total RNA was prepared from spleen, bone marrow, and intestine of the sacrificed animal, and linked VL-VH region gene pairs were generated by RT-PCR. The linked VL-VH region genes were ligated into the phh3mu-γ1 vector [2] to generate the phh3-VL-VH library [see Fig. (2)], resulting in an 'original' library of about 2×10^7 members. For expression of Fab on the phage surface, the prokaryotic bidirectional leader/promoter cassette containing the Zeocin™ selectable marker (*PZeoP*) was inserted between VL and VH, generating the Fab phage display library of vectors [phh3-VL-*PZeoP*-VH, see Fig. (2)]. The Zeocin™ selectable marker ensures that only vector molecules that have incorporated the cassette are perpetuated during library construction.

To select a Fab phage display sublibrary that reacts with the BT-20 carcinoma cells, phage (10^{11} cfu) were subjected to two consecutive rounds of panning on fixed BT-20 cells (with amplification of the phage by infection of bacteria after each pan). The first pan yielded 8×10^4 cfu of bound/eluted phage whereas the

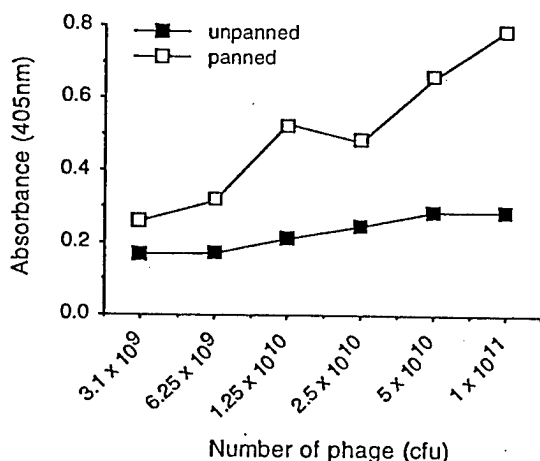
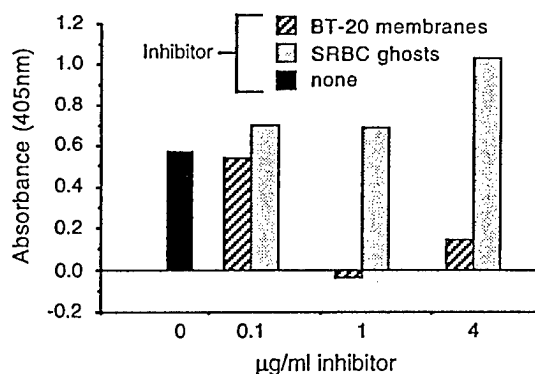


Fig. (4). ELISA of the mouse anti-BT-20 phage on BT-20 cells. As negative control, phage encoding no Fab on its surface was used.

second pan yielded 3×10^6 cfu of bound/eluted phage, a 37.5-fold increase over the first pan. This sequential enrichment is comparable to that seen by others [16]. The specificity of the panned Fab phage display library was shown by ELISA on fixed BT-20 cells [Fig. (4)]. Both the unpanned and panned libraries were determined to be polyclonal by nucleotide sequence analysis of the VL and VH region genes of individual clones ([23], data not shown).

To confirm the specificity of the panned Fab phage library for BT-20 cells, the library was tested in a "criss-cross" inhibition ELISA in conjunction with a panned anti-SRBC Fab phage library [24]. As shown in Fig. (5), BT-20 membranes inhibited the binding of the anti-BT-20 phage library to solid phase BT-20 cells in a dose-dependent manner [Fig. (5A)] but not of the anti-SRBC phage library to solid phase SRBC ghosts [Fig. (5B)]. Conversely, SRBC ghosts inhibited the binding of the anti-SRBC phage library to solid phase SRBC

A. Anti-BT-20 panned library



B. Anti-SRBC panned library

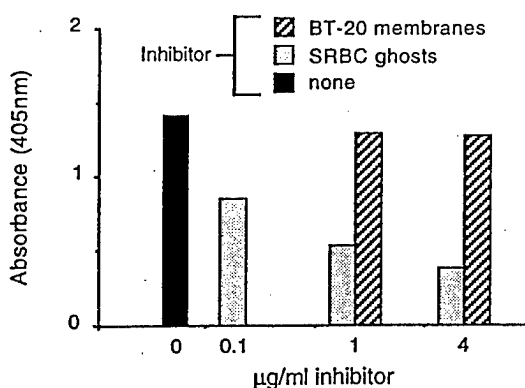


Fig. (5). Inhibition ELISA on A) BT-20 membrane-coated plate using the anti-BT-20 panned phage; and B) SRBC-coated plate using the anti-SRBC panned phage. (Increased binding is often seen in ELISAs at high potential inhibitor concentrations, presumably due to better binding at higher solute concentrations; hence the increased phage binding for both the BT-20 membranes and SRBC ghosts at 4 μg).

ghosts in a dose-dependent manner [Fig. (5B)] but not of the anti-BT-20 phage library to solid phase BT-20 cells [Fig. (5A)]. Thus, both the anti-BT-20 and anti-SRBC libraries showed specificity for their immunizing/panning polyantigens.

The results presented here demonstrate that a polyclonal Fab phage display library specific for human breast carcinoma cells can be generated using our bidirectional vector system. Such libraries, after suitable negative selection to eliminate major reactivities against normal tissue, could be transferred in mass to our bidirectional mammalian expression vector for production of libraries of whole antibodies [2] with C regions of any desired isotype or species. Specifically, chimeric antibodies with mouse V regions and human C regions would be useful for passive immunotherapy in humans. Polyclonal antibody libraries will mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and are expected to be useful for breast cancer therapy, as well as diagnosis.

Acknowledgments

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Abbreviations

AP	=	Alkaline phosphatase
C	=	Constant
cfu	=	Colony forming units
H chain	=	Heavy chain; i.p., intraperitoneally
L chain	=	Light chain
PP/	=	Leader-promoter-promoter-leader
PBS	=	Phosphate-buffered saline
pfu	=	Plaque forming units
RT-PCR	=	Reverse transcription - polymerase chain reaction
SRBCs	=	Sheep red blood cells
V	=	Variable
Zeo,	=	Zeocin™ resistance gene

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Recombinant Polyclonal Antibody Libraries

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Abstract: We describe a technology for generating recombinant polyclonal antibody libraries (PCALs) that enables the creation and perpetuation of standardized mixtures of polyclonal whole antibodies specific for a multiantigen (or polyantigen). Therefore, this technology combines the advantages of targeting multiple antigenic determinants -- high avidity, low likelihood of antigen 'escape variants', and efficient mediation of effector functions, with the advantages of using monoclonal antibodies -- unlimited supply of standardized reagents and the availability of the genetic material for desired manipulations. The technology for generating recombinant polyclonal antibody libraries begins with the creation of phage display Fab (antibody) libraries. This is followed by selection of sublibraries with desired antigen specificities, and mass transfer of the variable region gene pairs of the selected sublibraries to a mammalian expression vector for generation of libraries of polyclonal whole antibodies. We review here our experiments for selection of phage display antibody libraries against microbes and tumor cells, as well as the recent literature on the selection of phage display antibody libraries to multiantigen targets.

INTRODUCTION

The antibody response of vertebrates to most antigens is polyclonal and targets multiple epitopes on the antigen surface [1,2]. This characteristic has co-evolved with the effector mechanisms mediated by the Fc regions of antibodies -- such as complement binding with production of C3b, opsonization/phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC) -- that serve to eliminate the antigen [1-4]. Thus, triggering of effector functions requires a high density of antibodies on the antigen surface for sufficient cross-linking of Fc receptors on effector cells and for efficient C1q binding [5-7]. When the immunogen is a living cell, such as a microbe or a tumor cell, the polyclonality of the antibody response helps guard against the development of cell 'escape variants' that are no longer recognized by the antibodies. This is because the chance that a given cell will simultaneously lose all the target epitopes is (in most cases) essentially zero.

Passive immunotherapy with serum-derived polyclonal antibodies has been used for many years. The use of polyclonal human immunoglobulin (human gamma globulin) -- for the

treatment of patients with agammaglobulinemia, immunocompromised patients, and patients with severe microbial infections -- is generally effective and shows no adverse reactions [8-13]. Treatment of mice with serum-derived murine polyclonal antibodies has been shown to prevent development of a murine tumor [14]. Furthermore, occasional successful treatment of human malignancies, such as melanoma and renal cell carcinoma, with polyclonal antibodies derived from animals or from humans has been reported [15,16].

Despite their efficacy in many applications, the use of conventional polyclonal antibodies is limited by: short supply of serum antibody from immunized animals or from humans; inability to modify the antibodies because the genes are not available; and, in cases where a human cell population (malignant or normal) is targeted, the likely loss of the vast majority of the antibody population if negative selection were performed to eliminate major cross-reactivities with other tissues or cell populations. This is because many cell surface antigens are expressed on many different cell types.

Unlike polyclonal antibodies, monoclonal antibodies provide an unlimited supply of standardized reagents [17-19], and their heavy (H) and light (L) chain genes can be readily cloned and manipulated. However, monoclonal antibodies target single epitopes. Therefore, they are much less efficient at activating effector mechanisms than

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polyclonal antibodies (hence the need for toxic tags in therapeutic applications [20-23]. Furthermore, the likelihood of cell 'escape variants' -- that are no longer recognized by the antibody -- is much higher for monoclonal than polyclonal antibodies.

To combine the advantages of polyclonal and monoclonal antibodies, we have developed a technology for generating polyclonal antibody libraries. This technology enables the perpetuation of standardized mixtures of polyclonal antibodies specific for an antigen or a multiantigen target (a polyantigen). Hence, it combines the advantages of targeting multiple antigenic determinants (high avidity, low likelihood of antigen 'escape variants', and efficient mediation of effector functions) with the advantages of using monoclonal antibodies (unlimited supply of standardized reagents, and the availability of the genetic material for desired manipulations such as the replacement of mouse constant regions by human constant regions to generate chimeric antibodies [24,25].

The generation of polyclonal antibody libraries with desired specificities is made possible by the technology for displaying Fab antibody fragments on the surface of phage particles to generate so called "Fab phage display libraries" [26-29]. In this system the genetic material encoding Fab fragments is obtained from the B lymphocytes of immunized animals (or from humans) and cloned into a phagemid vector such that the genetic material encoding the Fab is attached to the genetic material encoding a phage coat protein. (Fab is comprised of 2 chains: the L chain and the Fd chain. The L chain contains the variable [V] and constant [C] region [VL-CL]; and the Fd chain contains the V region and the first H chain constant domain [VH-CH1].) On transformation of bacteria with these phagemid vectors (and superinfection with helper phage), phage particles that display Fab on their surface are generated because the Fab is embedded in the phage coat via the coat protein to which it is attached. Because each phage particle contains a different vector molecule for its genome, each phage particle displays a different Fab on its surface. Phage libraries larger than 10^8 different members can be generated. The libraries can be selected for binding to specific antigens (positive selection) by affinity chromatography [30-32], including panning on antigen-coated surfaces [29].

The beauty and power of the phage display system is the coupling of a selectable function (binding to an antigen) to the genetic material that encodes that function. This is because the selected phage have a replication function and therefore a

few phage particles can be amplified indefinitely if they are used to infect the bacterial host. This powerful system has been used by other investigators to select and clone-out phage encoding monoclonal antibody fragments to haptens, proteins, viruses, and cancer cells [33-42] as an alternative to the hybridoma technology.

In contrast, our laboratory has adapted the Fab phage display system to generate polyclonal antibodies (PCALs, pronounced "P"- "Cals") [43-47]. Instead of isolating monoclonal Fab phage particles, the Fab phage display libraries are maintained as polyclonal mixtures and subjected to positive and negative selection; positive selection to recover those phage particles with desired antigen specificities, and negative selection to remove those phage particles with undesired specificities (for example those with certain cross-reactivities). After amplification of the selected sublibraries, the selected antibody genes are transferred, in mass, from the phage vector population to a mammalian vector that provides complete constant region genes and appropriate transcription regulatory elements for expression of whole, glycosylated IgG antibodies in mammalian cells. Thus, the library that was selected at the Fab phage display level can be expressed -- after transfection of the mammalian vector population into mammalian cells -- as a library of polyclonal whole antibodies that can mediate effector functions. Because the transfected cells are immortal, the cell population producing the IgG polyclonal library can be perpetuated indefinitely, just like hybridoma cells. Alternatively, this cell population can be recreated as desired by transfection of the mammalian vector population into mammalian cells [Fig. (1)].

Polyclonal antibody libraries can be manipulated to eliminate undesirable specificities and have the unique advantage (not shared by conventional polyclonal antibodies) that desired specificities can be amplified. Thus, even if 99.99% of the antibody population to cancer cells is lost by absorption with normal cells, a library of 10^8 members will be reduced to 10^4 members, which could be amplified to generate unlimited supplies of the (still large) sublibrary. By comparison, a conventional polyclonal antibody preparation will be essentially lost after absorption to remove major reactivities against normal human cells.

PCALs (obtained by mass antigen-selection and mass transfer of selected variable region gene pairs without isolating or characterizing individual library members) differ from collections of monoclonal antibodies (each of which had been selected for

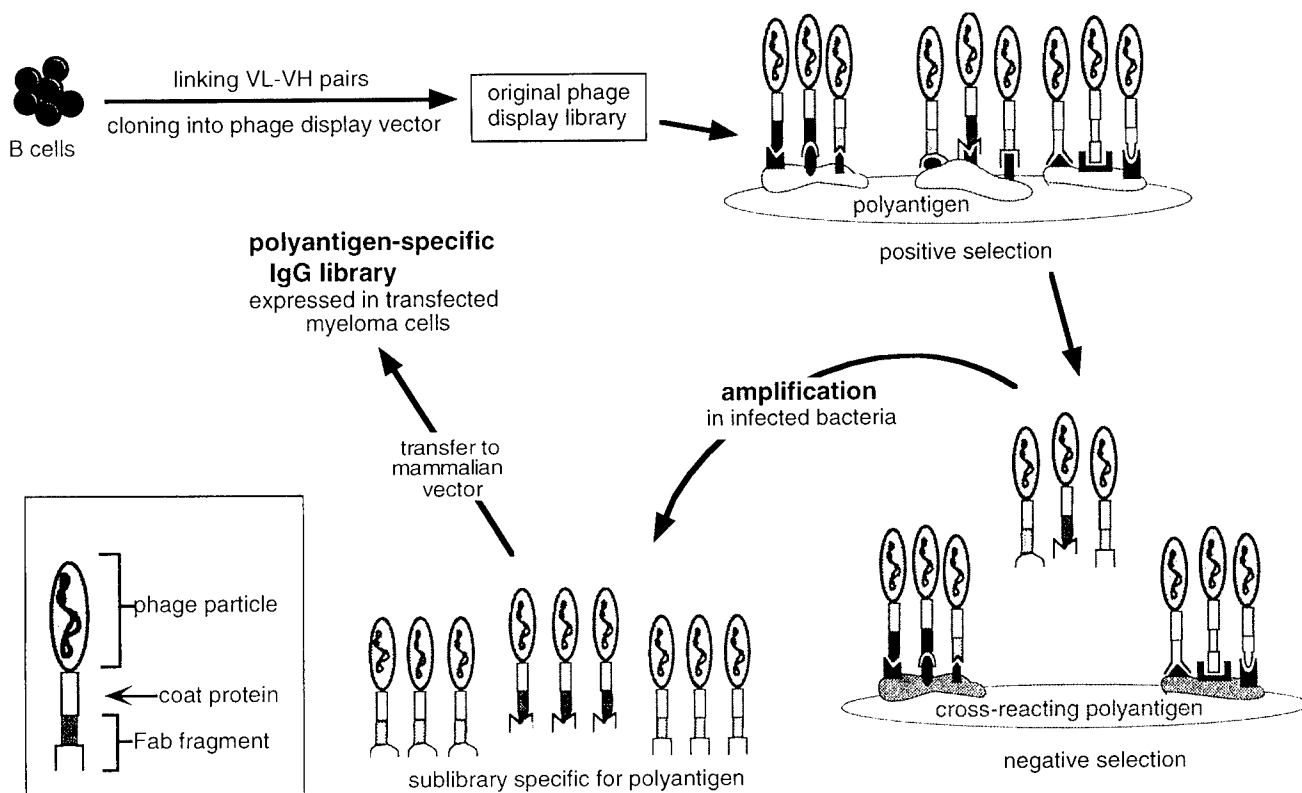


Fig. (1). Scheme for production and selection of a polyantigen-specific polyclonal antibody library.

antigen specificity). This is because PCALs contain individual antibodies which may or may not be specific for the target, but the collection of the individual antibodies recognizes the target antigen way above the background of any cross-reacting

antigen, with a high signal-to-noise ratio. This concept is exemplified schematically in Fig. (2).

THE SYSTEM FOR GENERATION OF PCALs

We have developed a system of phagemid and mammalian bidirectional vectors that facilitates the mass transfer of linked VL-VH region gene pairs [43,44], from antigen-selected sublibraries, without loss of the VL-VH combinations. The key feature of the transfer is that the VL and VH region genes are linked head-to-head ($\leftarrow\rightarrow$) in opposite transcriptional orientations. Linking of VL and VH region genes is done by reverse transcription - polymerase chain reaction (RT-PCR).

Primers, cDNA Synthesis, and VL-VH cDNA Linking

A method for cDNA synthesis and VL-VH cDNA linking was developed, and appropriate V and C region primers were designed [44,45]. In this method, RNA is obtained from B cell and plasma cell-containing tissues of immunized mice and used to prepare combinatorial libraries of VL-VH region gene pairs, linked head-to-head ($\leftarrow\rightarrow$)

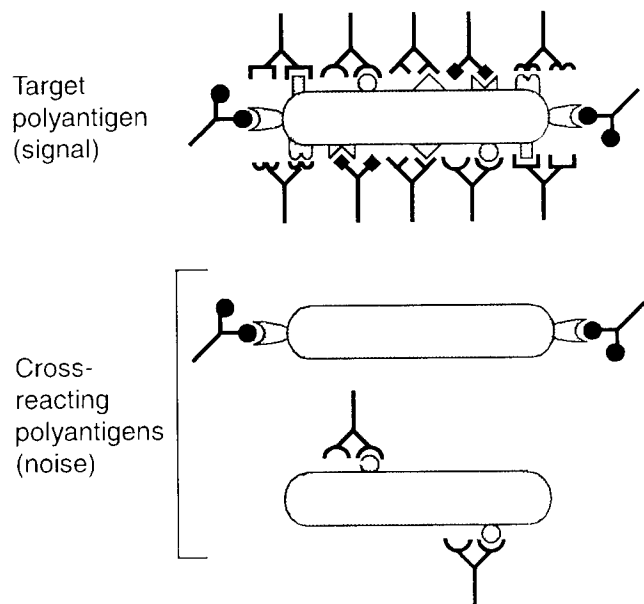


Fig. (2). Schematic representation of target recognition with high "signal-to-noise" ratio. (Although not shown, the cross-reacting polyantigens have additional antigens on their surface, that are not shared with the target polyantigen.)

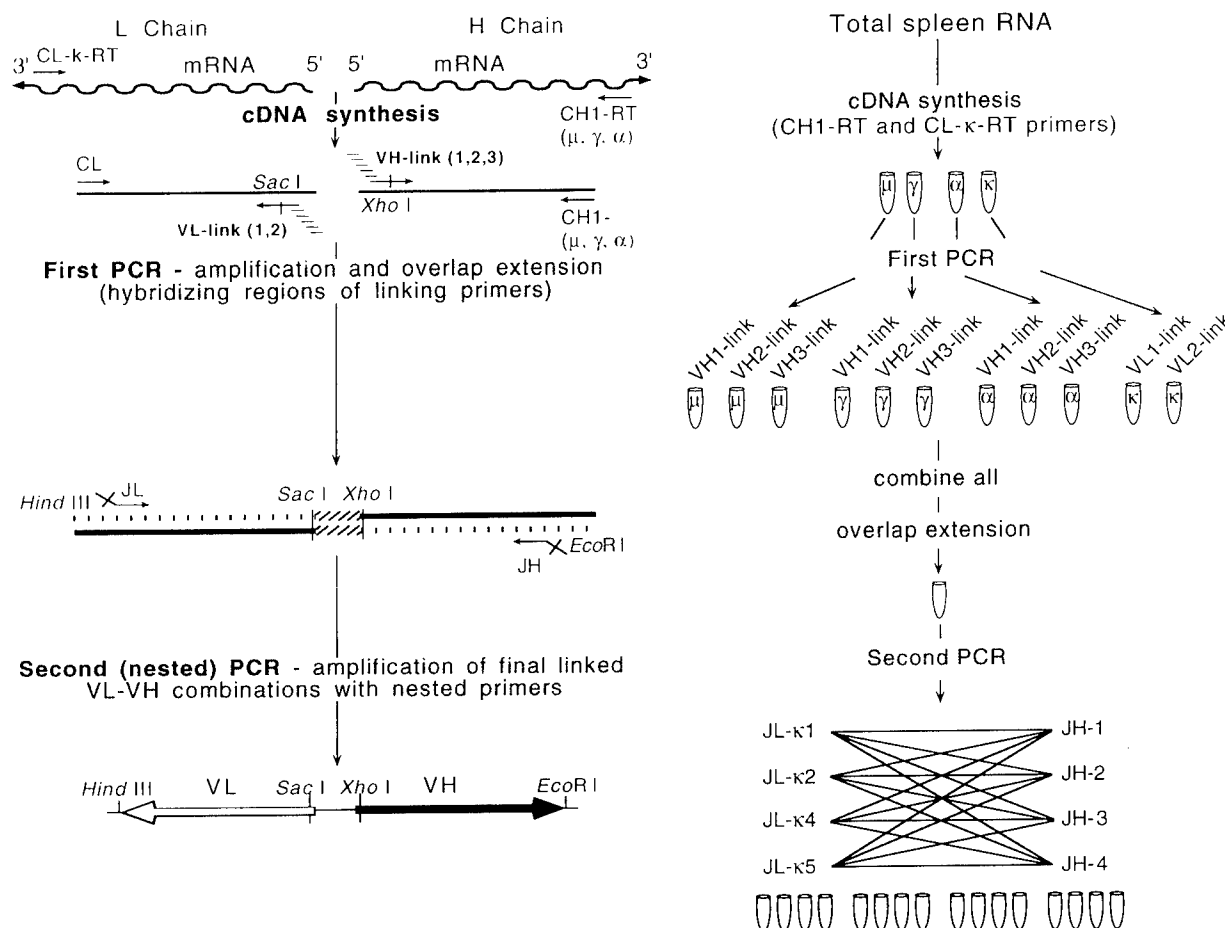


Fig. (3). General scheme for linking and amplifying VL-VH region gene pairs in opposite transcriptional orientations. VL-VH products are obtained after cDNA synthesis and consecutive PCR steps. See Fig. (4) for primer sequences.

in opposite transcriptional orientations. The general scheme for RT-PCR and linking of VL-VH region gene pairs is shown in Fig. (3). First strand cDNA synthesis of VL and VH region genes is done in 4 different tubes, each containing a different reverse primer hybridizing to μ H chains, to γ H chains (of all four mouse γ isotypes), to α H chains, or to κ L chains. The first strand cDNA library from each of the four tubes is then used in 3 (H chain) or 2 (L chain) PCRs using μ , γ , α , or κ nested reverse primers and a forward primer that hybridizes to the 5' end of VL or VH region genes. Each VH or VL primer provides a tail (link) for VL-VH linking that includes a restriction enzyme site (*Sac* I and *Xho* I for the 2 VL and 3 VH primers, respectively) necessary for subsequent insertion of promoter and leader sequences. The tail of VL primers is complementary to the tail of VH primers [see Fig. (4) for primer sequences]. Because of the relatively large number of mismatches (to prospective templates) in this minimal set of V primers, the First PCR is done under very low stringency conditions (37°C). However, subsequent steps

involve PCR using nested primers at high stringency to ensure that only Ig sequences are amplified [45].

The PCR-amplified L and H chain sequences are combined into a single tube, to allow hybridization of the complementary VL and VH tails and overlap extension to generate head-to-head-linked VL-VH region gene pairs. The product of the overlap extension reaction is then used in an array of 16 separate PCRs, each amplified with a different pair of JL and JH primers that hybridize to the four J κ and four JH mouse genes and which contain a *Hind* III or *Eco*R I site for the JL and JH primers, respectively [see Fig. (3) for scheme and Fig. (4) for primer sequences].

Generation of Fab Phage Display Libraries in a Bidirectional Phagemid Vector

A bidirectional phagemid vector was generated by modification of the unidirectional Fab phage

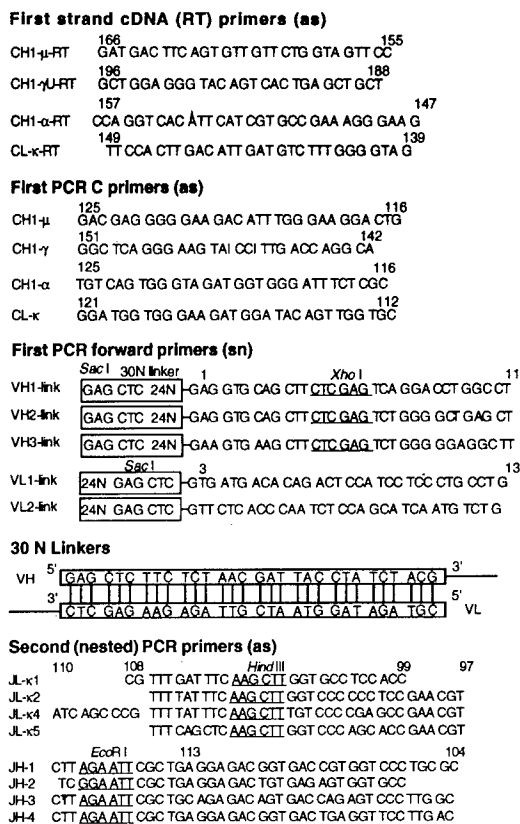


Fig. (4). Oligonucleotide primers used for library construction. The antisense and sense strands are denoted by "as" and "sn", respectively. Restriction sites are underlined. Corresponding amino acid numbers in the Kabat system [77] are shown above the sequences. Linker regions (the 30 nucleotide tails plus the first nucleotide of the VL or VH region) are indicated. N, nucleotide(s).

display vector pComb3 [29]. The bidirectional vector [48 phh3mu- γ 1, Fig. (5a)] contains, in opposite transcriptional orientations, a mouse C κ gene and a mouse (γ 1) CH1 gene segment attached to DNA encoding the carboxyl terminal end of the phage coat protein cpIII [44]. VL-VH region gene pairs linked head-to-head, in opposite transcriptional orientations, are cloned between the *Hind* III and *Eco*R I sites of the phh3mu- γ 1 vector [Fig. (5a)] to generate a library of vectors [phh3VL-VH-lib, see Fig. (5b)]. Because the VL and VH region genes, in each pair, are separated by *Sac* I and *Xho* I restriction enzyme sites, a cassette containing head-to-head bacterial promoter (P) and leader (l) sequences is obtained from vector #560 pIPZeoPl [Fig. (5b)] and cloned between the *Sac* I and *Xho* I sites of phh3VL-VH-lib to drive expression of κ chain and Fd-cpIII (VH-CH1-cpIII) proteins. This generates a library of Fab phage display vectors [phh3-VL-IPZeoPl-VH-lib, Fig. (5c)]. The bacterial promoter-leader cassette in vector #560 pIPZeoPl had been generated from

phagemid vector pComb3 [29], by a series of modifications [44,47]. The cassette contains the *lacZ* and *tac* promoters to drive expression of the κ and Fd-cpIII chains respectively. Although the leader sequences for both chains have the *pelB* leader amino acid sequence, the nucleotide sequences differ at many positions [44], and therefore, deletions are unlikely to occur during replication of the vector. The bacterial promoter-leader cassette also includes the selectable marker ZeocinTM [49,50] (driven by the synthetic promoter EM-7), which ensures that only vector molecules that have incorporated the cassette are perpetuated during library construction [47].

Transformation to produce colonies and infection of bacteria to produce phage display libraries are done as described [29]. The Fab phage display libraries can be selected for binding to polyantigens, to generate specific sublibraries (described in subsequent sections).

Mass Transfer of VL-VH Region Gene Pairs from the Phagemid Vector to a Bidirectional Mammalian Expression Vector

Generation of polyclonal whole antibodies requires the mass transfer of selected VL-VH region gene pairs from the phagemid vector to a mammalian expression vector, without loss of the VL-VH combinations. Therefore, a bidirectional mammalian vector for expression of mouse IgG2b antibodies was constructed ([43] and C.-Y. Kao and J. Sharon, manuscript in preparation). This vector, denoted #577 pMDV-IgG2b [Fig. (6), right side] contains the mouse γ 2b heavy chain C gene and the mouse κ light chain C gene in opposite transcriptional orientations, as well as the *gpt* selectable marker, and mammalian transcription termination and polyadenylation sites.

VL-VH region gene pairs linked head to head ($\leftarrow\rightarrow$) in opposite transcriptional orientations, are transferred, in mass, between the (circular) bidirectional phage display vector and the (circular) mammalian expression vector. The transfer from a phage display to a mammalian expression vector requires opening the phage display vector with *Sac* I and *Xho* I [Fig. (6a)] between the VL and VH amino termini and replacing the prokaryotic IPZeoPl cassette with a mammalian lPEHP/m cassette (which contains Ig promoter and leader sequences in opposite orientations as well a heavy chain enhancer), yielding the intermediate vector shown in Fig. (6b). The VL-VH pairs including

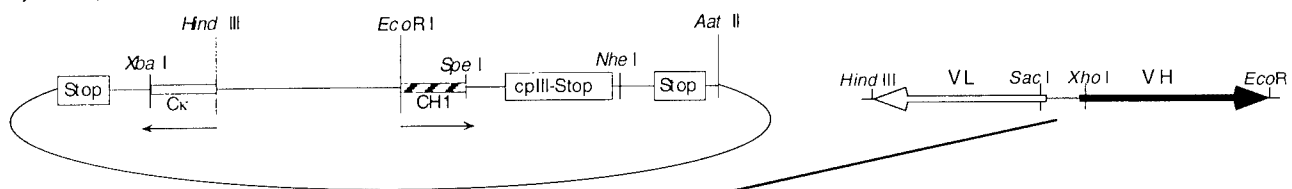
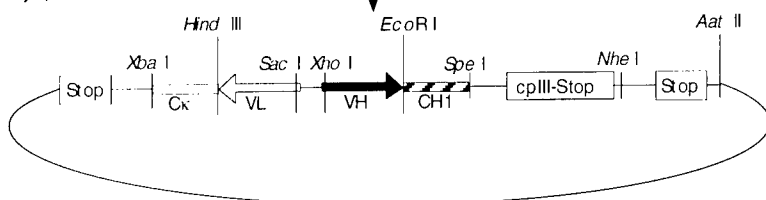
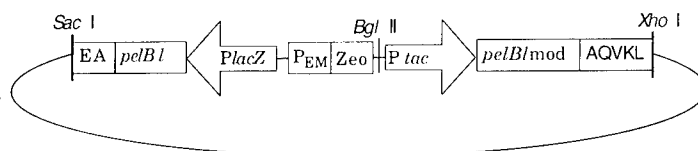
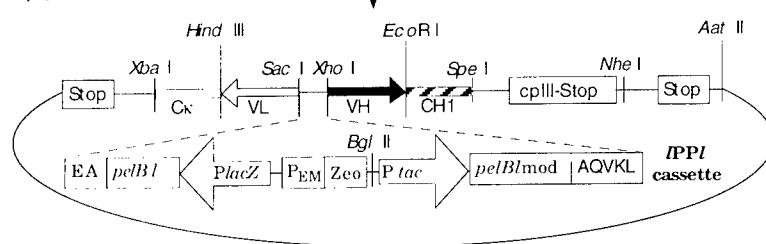
a) #541 phh3mu- γ 1**b) phh3-VL-VH-lib****#560 pPZeoPI****c) phh3-VL-IPZeoPI-VH-lib**

Fig. (5). Scheme for construction of Fab phage display libraries (partial maps and not to scale); all vectors are circular. The direction of transcription is indicated by arrows and by arrow heads on promoters and variable region genes.

Symbols: P, promoter; L, leader sequence; Lmod, leader sequence with modified nucleotide sequence; Stop, translation termination; Zeo, gene for ZeocinTM selectable marker. Amino acids encoded by the vectors are shown in one letter code.

the mammalian lPEHP/m cassette are then cut from the intermediate vector [Fig. (6b)] with *EcoR* I and *Hind* III and cloned into the *EcoR* I - *Hind* III sites of the pMDV-IgG2b mammalian vector, to generate pMDV-IgG2b-lib [Fig. (6c)]. (In the original design [44], the VL-VH region gene pairs including a mammalian promoter-leader cassette were lifted by PCR, and inserted into a mammalian vector. This step has subsequently been modified to use restriction enzyme-based transfer).

TESTING OF THE SYSTEM FOR GENERATING POLYCLONAL ANTIBODY LIBRARIES

The functionality of the bidirectional phage display and mammalian expression vectors as well as the generation and transfer of linked VL-VH

region gene pairs were tested [43,44]. In one series of experiments [44], V region gene pairs derived from an A/J mouse that had been immunized with the hapten *p*-azophenylarsonate (Ars) coupled to keyhole limpet hemocyanin (KLH) were selected for Ars-binding in the phage display vector, transferred to the mammalian vector, and expressed as a mouse IgG2b PCAL. As expected, individual IgG2b antibodies from the PCAL had V region sequences and Ars-binding characteristics similar to those of anti-Ars hybridomas [44].

GENERATION OF POLYCLONAL FAB PHAGE DISPLAY LIBRARIES TO POLYANTIGENS

Using our system for PCAL generation, described above, we have generated Fab phage

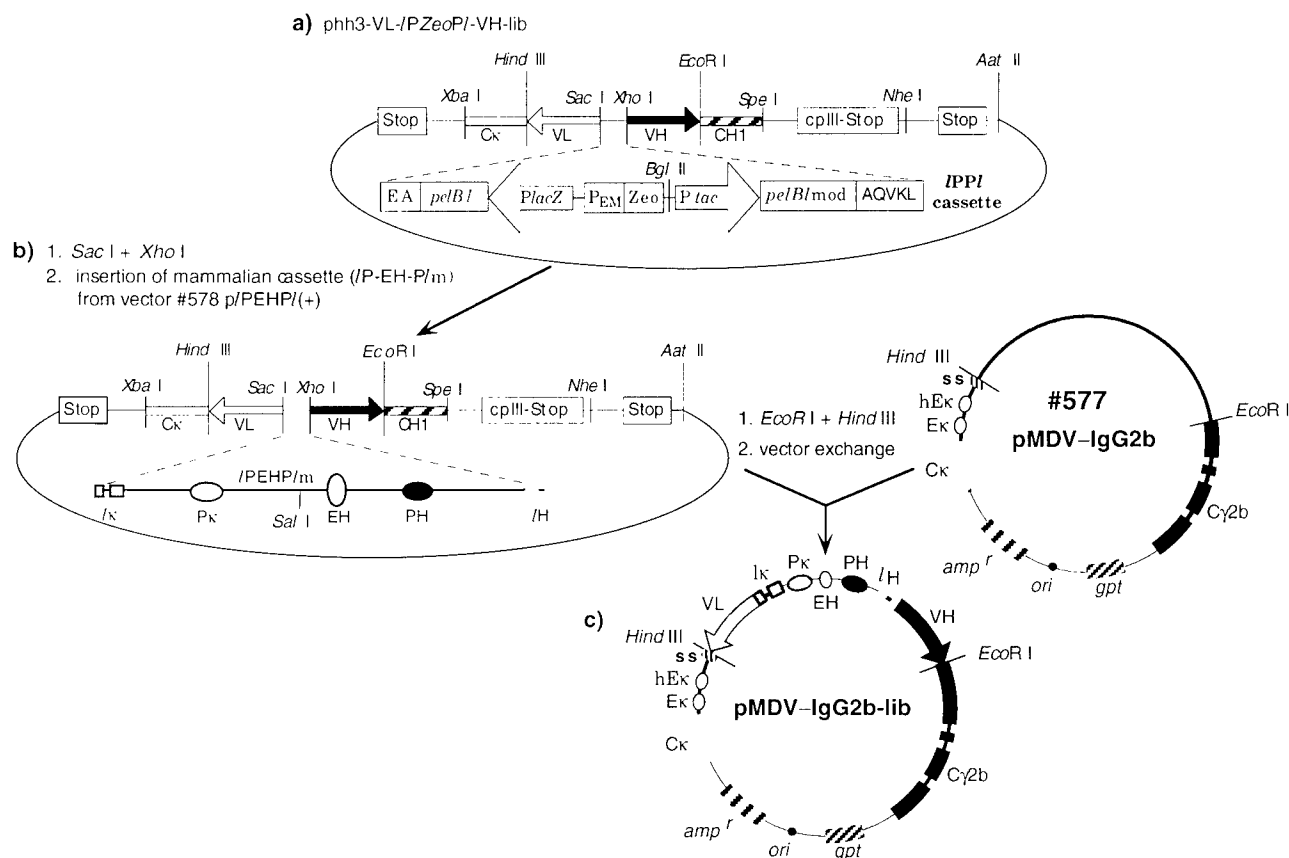


Fig. (6). Transfer of V region gene pairs between bidirectional phage display and mammalian expression vectors (partial maps and not to scale).

Symbols: Prokaryotic elements are as in Fig. (5). Mammalian regulatory elements are oval shaped. amp^r, ampicillin resistance; ori, prokaryotic origin of DNA replication; P, promoter; E, enhancer; I, leader sequence; ss, splice site; hum, human (all other mammalian regulatory elements are murine).

display libraries to infectious agents and cancer cells.

Generation of a Polyclonal Fab Phage Display Library to the Protozoan Parasite *Cryptosporidium parvum*

Cryptosporidium parvum is a protozoan parasite that causes severe disease in AIDS patients, for which there is no effective treatment [46]. A Fab phage display library was constructed from the RNA of spleen, intestine, nasopharynx, and bone marrow of *C. parvum*-immunized BALB/c mice. The Fab-displaying phage library was selected for binding to an oocyst/sporozoite preparation of *C. parvum*, generating an anti-*C. parvum* Fab phage display library of 5.2×10^4 members, which was tested for antigen-binding by direct ELISA. As shown in Fig. (7), the *C. parvum*-selected phage library bound to a *C. parvum*-coated plate whereas the unselected library and a negative control of

phage containing the bidirectional phage display vector without the Fab gene sequences did not bind [46].

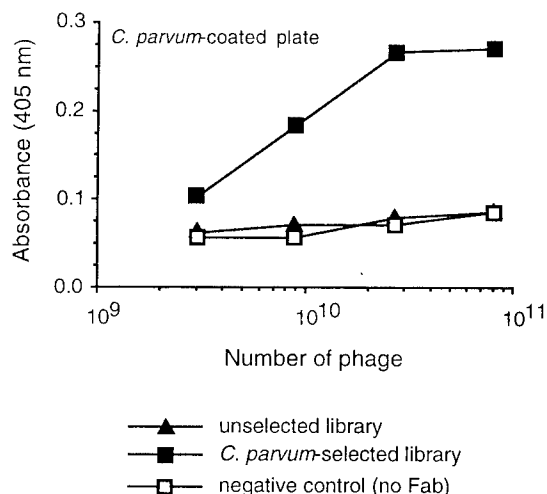


Fig. (7). Specificity of anti-*C. parvum* Fab phage display library shown by direct ELISA.

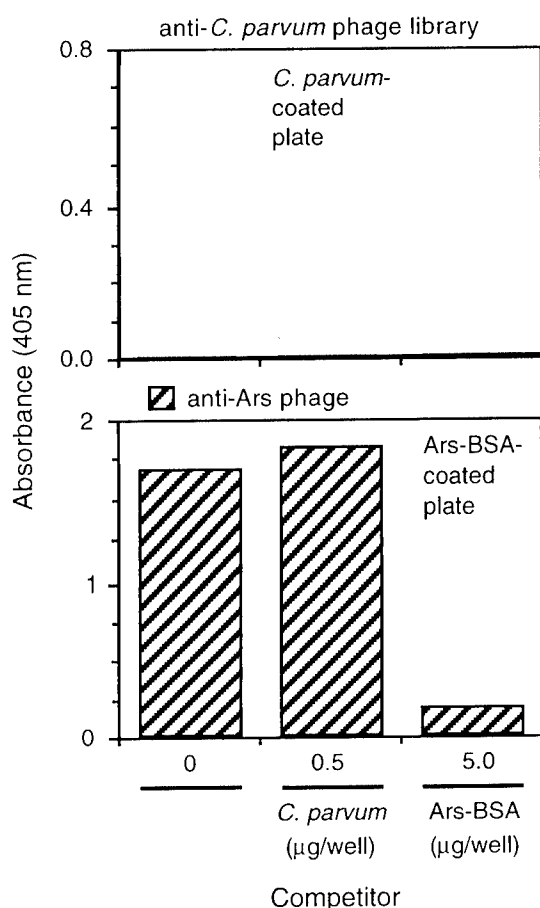


Fig. (8). Specificity of anti-*C. parvum* Fab phage display library shown by inhibition ELISA.

The specificity of the *C. parvum*-selected phage library was further demonstrated by a criss-cross inhibition ELISA using both the anti-*C. parvum*-selected phage and phage displaying Fab specific for the Ars hapten. As seen in the top panel of Fig. (8), the binding of the anti-*C. parvum* phage to solid phase *C. parvum* was inhibited by preincubation with the oocyst/sporozoite preparation but not by preincubation with Ars coupled to bovine serum albumin (BSA). Conversely, binding of the anti-Ars phage to solid phase Ars-BSA was inhibited by preincubation with Ars-BSA but not by preincubation with the oocyst/sporozoite preparation of *C. parvum* [see bottom panel of Fig. (8)].

Restriction fragment length polymorphism (RFLP) analysis and nucleotide sequencing of several members of the *C. parvum*-selected library indicated that the selected library is diverse.

Generation of Fab Phage Display Libraries to Human Ovarian Tumor Cell Membranes and Sheep Red Blood Cells

Cells from a surgically-resected human ovarian carcinoma (designated OC2), and sheep red blood cells (SRBCs) as control, were separately used to immunize mice [45]. BALB/c mice were immunized with either OC2 membranes or SRBCs by 3 intraperitoneal injections in complete Freund's adjuvant. RNA samples obtained from the spleens of the immunized mice were separately used to prepare Fab phage display libraries, using the system and primers described above. Each library was subjected to two consecutive pannings on wells coated with the immunizing polyantigen (OC2 or SRBC membranes, each library in two wells of a 24-well plate). Direct binding solid phase ELISA of the anti-OC2 and anti-SRBC Fab phage display libraries showed that successive panning increased specificity for the panning membranes [45]. Furthermore, binding of the second OC2 panned library to an OC2 membrane-coated plate was specifically inhibited by OC2 membranes but not by SRBC membranes. Conversely, binding of the second SRBC panned library to an SRBC membrane-coated plate was specifically inhibited by SRBC membranes but not by OC2 membranes [45].

Generation of a Fab Phage Display Library to the Human Breast Cancer Cell Line BT-20

A polyclonal Fab phage display library to BT-20 cells was constructed from RNA of spleen, bone marrow, and intestine from a BALB/c mouse that had been immunized with BT-20 cells [47]. To select an Fab phage sublibrary that reacts with the BT-20 carcinoma cells, phage were subjected to two consecutive rounds of panning on fixed BT-20 cells. The first pan yielded a library of 8×10^4 members, whereas the second pan yielded a library of 3×10^6 members. Analysis of the unpanned and panned Fab phage libraries by ELISA on fixed BT-20 cells showed that the second pan library binds better than the first pan library which, in turn, binds better than the unpanned library [47].

To confirm the specificity of the second pan Fab phage library for BT-20 cells, the library was tested in a criss-cross inhibition ELISA in conjunction with the panned anti-SRBC Fab phage library. BT-20 membranes inhibited the binding of the anti-BT-20 phage library to solid phase BT-20 cells in a

dose-dependent manner but not of the anti-SRBC phage library to solid phase SRBC membranes. Conversely, SRBC membranes inhibited the binding of the anti-SRBC phage library to solid phase SRBC membranes in a dose-dependent manner but not of the anti-BT-20 phage library to solid phase BT-20 cells [47].

RECENT LITERATURE ON SELECTION OF PHAGE DISPLAY ANTIBODY LIBRARIES TO MULTIANTIGEN TARGETS

Selection of phage displaying antibody fragments with desired antigen specificity, from a phage display antibody library with no demonstrable specific binding to the desired antigen(s), can be achieved by any method that separates antigen-binding phage from non-antigen binding phage. Commonly, antigen is immobilized on a solid surface and phage incubated with the antigen to capture binding phage, a method known as panning [29,33,51,52]. The solid surface is washed to remove non-binding phage, the binding phage are eluted and used to infect bacteria, resulting in amplification of the antigen-selected phage. Elution of bound phage can be achieved by any of several methods: using acidic solutions such as HCl-glycine [51], with basic solutions like triethylamine [33], by competition with antibodies to the antigen [53], or by enzymatic cleavage of a protease site engineered between the antibody fragment and the cpIII protein [54]. Alternatively, direct bacterial infection without chemical elution of phage has been used [55]. Sequential rounds of selection are often performed to obtain a high percentage of antigen-specific phage [29,33,51-53,55-65].

For selection of antibody-displaying phage (often referred to as "phage antibodies") to a multiantigen target (i.e. polyantigen), such as microbial or mammalian cells, panning as well as other methods have been used. Several groups have reported the successful isolation of phage antibodies binding to microbes by selecting on whole microbes [46,56,57,66]. Monolayers of adherent cancer cells and cell lines transfected to express an antigen of interest, either fixed [47,58-60] or non-fixed [39,61,62], have been used to successfully isolate specific phage antibodies. Many groups have also used such cell lines in suspension to select for binding phage [42,63-65,67-71]. The methods in which the phage antibodies that bind to the target cells are separated from the non-binders are diverse: centrifugation to

pellet bound phage and target cells leaving non-binding phage in the supernatant [42,63-65], flow cytometry to isolate a specific cell population with bound phage [67,69,70], and magnetic beads to sort desired cell-binding phage [68,71]. Isolation of phage antibodies to cell surface antigens has also been reported by groups using cancer tissue sections on slides [72] and thymic tissue fragments [73].

These methods of selection are used to isolate binding phage (positive selection), however, one may wish to rid the library of phage antibodies that may cross-react with or have epitopes in common with another polyantigen (e.g. normal, non-cancerous cells). To achieve this one or more rounds of negative selection may be performed before or after positive selection, using normal cells (or any other polyantigen of choice) to absorb the unwanted cross-reactive phage antibodies [39,62,64]. A round of negative selection may be performed simultaneously with the positive selection by combining both positively selecting target cells (e.g. cancer cells) and negatively selecting absorber cells with the phage library, then separating the desired selecting target cells with binding phage from the absorber cells with binding phage [67,68]. Separation may be achieved with flow cytometry [67], magnetic beads [68], or any method to sort the target cells from the absorber cells. Once separated, only the phage bound to the target cells would be eluted.

There are many selection strategies one could use in the creation of polyclonal antibody libraries to polyantigens, as outlined above, but the strategy must be carefully designed because the composition of the output library of phage antibodies will depend on the method used. For example, one group's initial attempts to pan on confluent monolayers of transfected CHO cells yielded predominantly phage antibodies that were reactive with serum proteins. It was hypothesized that the serum proteins present in the cell culture medium adhered to the flask during cell growth and therefore were present during phage selection. The problem was overcome by a strategy that kept cells in suspension during selection [63].

In the same study it was also noted that as the number of selection rounds increased, the diversity of selected phage antibodies decreased, with certain clones dominating in later selection rounds [63]. The same problem was discussed at a recent antibody engineering conference, and it was pointed out that during selection procedures competition may cause loss of many potential

binders, resulting in output phage dominated by a few clones. This problem becomes even worse when using complex antigens such as whole cells. The presenting group solved the problem by doing only one round of selection [74]. Thus, for selection of polyclonal phage antibodies to polyantigens, it is especially important that the number of selection rounds be limited to one or two, as more selection rounds may restrict the size of the selected library.

CONCLUSION

The technology is now available for the production of polyclonal antibody libraries that will simultaneously recognize many epitopes on a multitude of antigens on tumor cells, microbes, or any other polyantigens, greatly reducing the chance of escape variants. These standardized antibody mixtures will be available in unlimited supply, will be amenable to alteration by genetic manipulations, and will be efficient at mediating effector functions to eliminate target polyantigens.

Regarding future FDA approval, PCALs should fall under the same category as such clinically used polyclonal antibody products as human gamma globulin preparations, anti-thymocyte globulin (ALG) and anti-thymocyte sera (ATS) [75,76]. Like these products, every PCAL batch will have to be tested for safety, but PCALs are expected to show much less batch-to-batch variability.

ACKNOWLEDGMENTS

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ABBREVIATIONS

Ars	= <i>p</i> -Azophenylarsonate
BSA	= Bovine serum albumin
C region or domain	= Constant region or domain
H chain	= Heavy chain

L chain	= Light chain
PCAL	= Polyclonal antibody library
PCR	= Polymerase chain reaction
RT	= Reverse transcription
SRBC	= Sheep red blood cell
VH	= Heavy chain variable region
VL	= Light chain variable region

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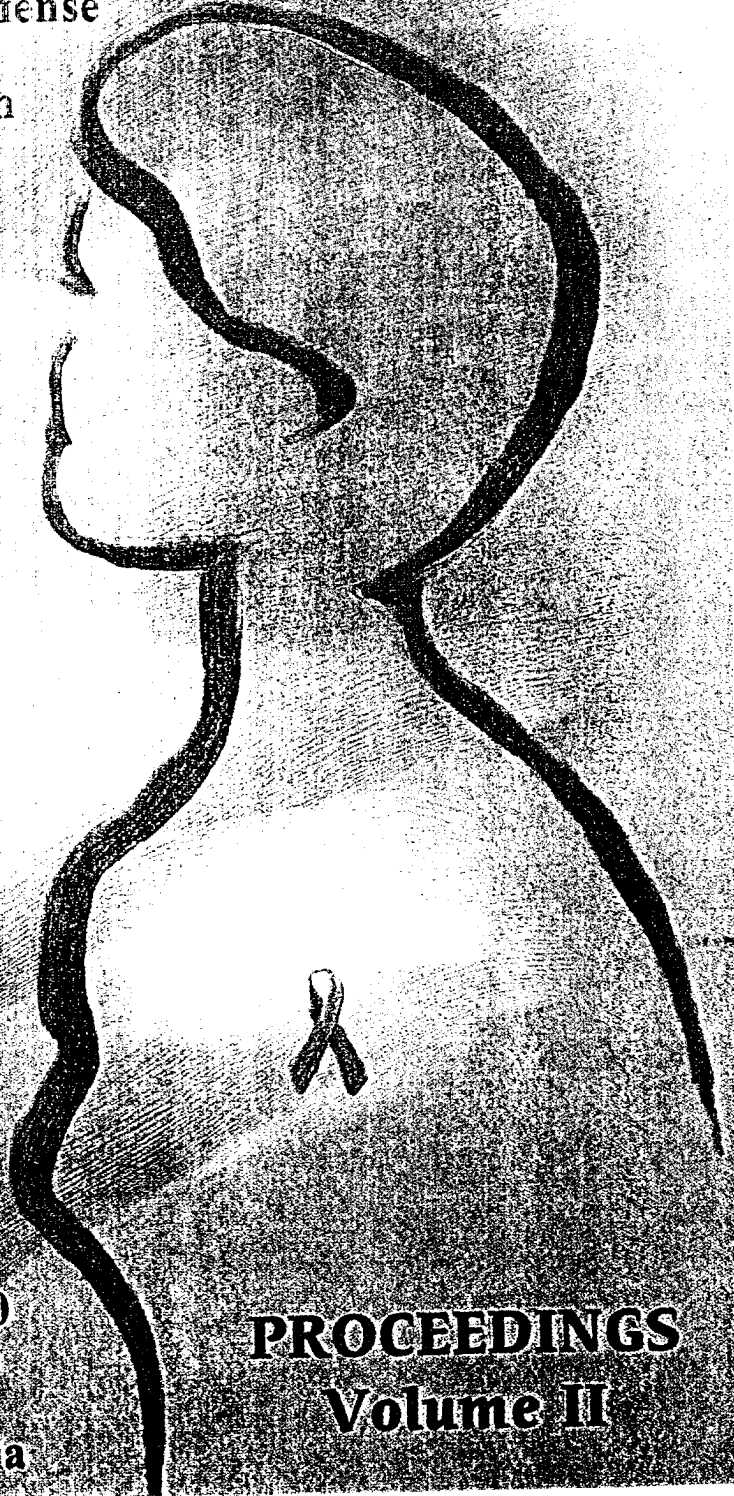
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Era of Hope

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PROCEEDINGS
Volume II

GENERATION OF A POLYCLONAL FAB PHAGE DISPLAY LIBRARY TO THE HUMAN BREAST CARCINOMA CELL LINE BT-20

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As an immunotherapeutic approach, we set out to develop a recombinant polyclonal antibody library that would target human breast cancer cells. Polyclonal antibody libraries are standardized mixtures of whole antibodies that can be perpetuated and modified as desired because the antibody genes are available. Therefore, they combine the advantages of conventional polyclonal antibodies (targeting of many antigenic determinants, low likelihood of antigen 'escape variants', and efficient mediation of effector functions) with the advantages of monoclonal antibodies (unlimited supply of standardized reagents, and the availability of the genetic material for desired manipulations).

We have previously described a vector system for generating recombinant polyclonal antibody libraries. This system uses bidirectional phagemid and mammalian expression vectors to facilitate mass transfer of selected variable light and variable heavy (VL-VH) region gene pairs from the phagemid to the mammalian vector, to express polyclonal libraries of whole IgG antibodies. We report here the first phase of generating a polyclonal antibody library to the human breast carcinoma cell line BT-20, using this vector system. VL and VH region gene pairs were obtained from a mouse immunized with BT-20 cells, and cloned, in opposite transcriptional orientations, in the bidirectional phagemid vector, to produce an Fab phage display library. This library was selected by panning on BT-20 cells and shown to be antigen-specific as demonstrated by inhibition ELISA (see Figure 1).

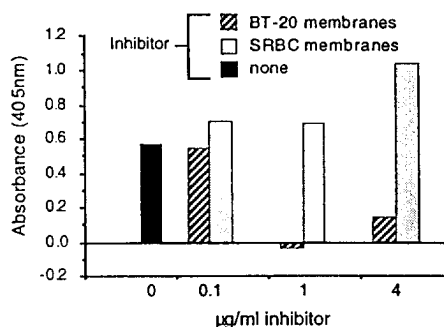


Figure 1. Inhibition ELISA on BT-20 membrane-coated plate using the anti-BT-20 panned phage. SRBC, sheep red blood cells.

We conclude that such libraries, after suitable negative selection to eliminate major reactivities against normal tissue, could be transferred in mass to our bidirectional mammalian expression vector for production of libraries of chimeric antibodies with mouse V regions and human constant (C) regions. These polyclonal antibody libraries will mediate effector functions, and are expected to be useful for breast cancer therapy as well as diagnosis.

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